Validation of an enzyme-linked immunoassay (ELISA) for plasma oxytocin in a novel mammal species reveals potential errors induced by sampling procedure

Robinson, Kelly J.; Hazon, Neil; Lonergan, Mike; Pomeroy, Patrick P.

Published in:
Journal of Neuroscience Methods

DOI:
10.1016/j.jneumeth.2014.01.019

Publication date:
2014

Journal of Neuroscience Methods 226 (2014) 73–79

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Basic Neuroscience

Validation of an enzyme-linked immunoassay (ELISA) for plasma oxytocin in a novel mammal species reveals potential errors induced by sampling procedure☆

Kelly J. Robinson[a,*], Neil Hazon[b], Mike Lonergan[a], Patrick P. Pomeroy[a]

[a] Sea Mammal Research Unit, Scottish Oceans Institute, University of St Andrews, St Andrews, Fife KY16 8LB, UK
[b] Scottish Oceans Institute, University of St Andrews, Scotland KY16 8LB, UK

HIGHLIGHTS

- An ELISA protocol for plasma oxytocin in phocid seals was validated.
- Sample handling protocols for accurately detecting plasma oxytocin were compared.
- Concentrations detected in raw plasma varied significantly with vacutainer type.
- Concentrations detected in extracted plasma were unaffected by vacutainer type.
- Capture and restraint protocol affected concentrations detected in raw plasma.

ARTICLE INFO

Article history:
Received 23 November 2013
Received in revised form 17 January 2014
Accepted 20 January 2014

Keywords:
Oxytocin
EDTA
Heparin
Extraction
Plasma
Seal

ABSTRACT

Background: The neuropeptide oxytocin is increasingly the focus of many studies investigating human and animal social behaviours and diseases. However, interpretation and comparison of results is made difficult by a lack of consistent methodological approaches towards analysing this hormone.
New method: This study determined the sample collection and analysis protocols that cause the least amounts of protocol dependant variation in plasma oxytocin concentrations detected by ELISA. The effect of vacutainer type, sample extraction prior to analysis and capture and restraint protocol were investigated while validating an assay protocol for two novel species, grey seals (*Halichoerus grypus*) and harbour seals (*Phoca vitulina*).
Results: Where samples are extracted prior to analysis, vacutainer type (EDTA mean: 8.25 ± 0.56 pg/ml, heparin mean: 8.25 ± 0.62 pg/ml, $p = 0.82$), time taken to obtain a sample and restraint protocol did not affect the concentration of oxytocin detected. However, concentrations of oxytocin detected in raw plasma samples were significantly higher than those in extracted samples, and varied significantly with vacutainer type (EDTA mean: 534.4 ± 43.7 pg/ml, heparin mean: 300.9 ± 19.6 pg/ml, $p < 0.001$) and capture and restraint methodology. There was no relationship between oxytocin concentrations detected in raw and extracted plasma ($p = 0.25$).
Comparison with existing method(s): Over half the reviewed published studies analysing plasma oxytocin use raw plasma and different vacutainer types are used without consistency or justification through-out the literature.
Conclusions: We caution that studies using raw plasma are likely to over estimate oxytocin concentrations, cannot be used to accurately infer true values via correlations and are susceptible to variation according vacutainer type.

1. Introduction

Oxytocin is a neuropeptide crucial for initiating and modulating maternal and social behaviour across vertebrate animals (Gimpl and Fahrenholz, 2001). Produced in the paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus, oxytocin is stored in the posterior pituitary gland until its release is triggered by a variety of stimuli, including scent, sight, touch and sound of a dependant infant or social partner (Uvnäs-Moberg, 1998;

* Corresponding author. Tel.: +44 01334462635.
E-mail addresses: kjr33@st-andrews.ac.uk, kelly.j.robinson@hotmail.co.uk (K.J. Robinson).

0165-0270/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jneumeth.2014.01.019

Neumann, 2008; MacDonald and MacDonald, 2010). Oxytocin is released both centrally into the brain and peripherally into circulation (Neumann and Landgraf, 2012) and it promotes care-giving and pro-social behaviours (Kosfeld et al., 2005; Ross and Young, 2009) by providing a physiological reward for their expression by activating reward and pleasure pathways in the brain (Baskerville and Douglas, 2010).

While its role in the physiology of parturition and lactation has long been known (Dale, 1906), oxytocin's effects on behaviour are still being explored. Studies looking to investigate peripheral levels of the hormone in relation to both animal and human behaviour have increased throughout the 21st century. In recent years there have been many studies examining possible links between oxytocin and a range of human psychological conditions, their causes and treatment including post-partum depression (Levine et al., 2007; Feldman et al., 2010a; Skrundz et al., 2011), anxiety (Hoge et al., 2012; Weisman et al., 2013), autism (Modahl et al., 1998; Hollander et al., 2003; El-Masry et al., 2010), depression (Scantamburlo et al., 2007; Holt-Lunstad et al., 2011), stress (Heinrichs et al., 2003; Grippo et al., 2009), anorexia nervosa (Hoffman et al., 2012) and suicidal behaviour (Deisenhammer et al., 2012). Oxytocin has also been used to investigate social and reproductive behaviour and physiology in a variety of domestic and laboratory mammalian species such as dogs (Odendaal and Meintjes, 2003), sheep (Lévy et al., 1995), primates (Amico et al., 1990; Maestripieri et al., 2009), mice (McCarthy, 1990), voles (Bales et al., 2007) and rats (Popik et al., 1992). However plasma oxytocin concentrations have never been determined in any mammalian species outside of laboratory, domestic or primate species, and the current study reports for the first time data from studies conducted on two species of phocis seals the grey seal (*Halichoerus grypus*) and harbour seal (*Phoca vitulina*).

Oxytocin has been assayed in various substrates including saliva (Carter et al., 2007; Feldman et al., 2010a,b), urine (Nagasawa et al., 2009; Moscovice and Ziegler, 2012; Crockford et al., 2013), cerebro-spinal fluid (Devarajan and Rusak, 2004; Martínez-Lorenzana et al., 2008), and milk (Leake et al., 1981; Prakash et al., 2009). However the most common medium used for oxytocin detection is plasma (of 50 papers reviewed using one or multiple substrates, $n = 39$ plasma, $n = 9$ saliva, $n = 8$ urine, $n = 6$ cerebro-spinal fluid, $n = 5$ serum and $n = 2$ milk). Despite its potential as a tool for investigating the expression of social and maternal behaviour in mammalian species, few analysis protocols for detecting oxytocin concentrations in plasma taken from wild populations or species have been validated.

The effect of capture and sampling procedure on detected plasma oxytocin concentrations of wild mammalian species has never been investigated in any prior study, and it is vital to ensure that abnormal concentrations of oxytocin are not being generated during this potentially stressful process. Oxytocin is documented to be released into circulation during extreme stressors such as restraint over an extended time period in rodents (Grippo et al., 2009; Pournajafi and Carter, unpublished observations) and is hypothesised to occur during procedures such as injections (Devarajan and Rusak, 2004). Therefore any attempt to study basal levels of this hormone in wild populations, which must be manually or chemically restrained during the sampling procedure, must be accompanied by investigation of whether oxytocin is released when sampling occurs.

Even with the increasing number of studies using plasma to investigate oxytocin, there is still contention about the appropriate way to collect and analyse samples accurately, and there has been a call for new research into reliable, accurate methodologies for investigators to utilise in future research (McCullough et al., 2013). The majority of studies published use an enzyme-linked immunosorbent assay (ELISA) kit manufactured by Assay Designs Inc. (Ann Arbor, MI, USA). However despite this manufacturer providing a protocol for collection and preparation prior to analysis, there is huge variation between studies in the methods researchers actually employ (of 39 papers, $n = 3$ unextracted serum, $n = 13$ extracted plasma, $n = 17$ unextracted plasma and $n = 6$ did not specify if extraction was used. For vacutainer type used in sampling, $n = 12$ ethylenediaminetetraacetic acid (EDTA) vacutainers, $n = 10$ lithium heparin vacutainers and $n = 14$ did not specify what vacutainer was used). In addition to the varying analysis protocols, there is disagreement in the literature over whether oxytocin concentrations measured in raw plasma are correlated with those in extracted samples, and how accurate the use of raw plasma is (McCullough et al., 2013). Currently some studies rely on the hypothesis that there is a direct relationship between oxytocin concentrations determined in extracted and raw samples (Hoge et al., 2012), provide data showing a correlation between oxytocin levels in raw and extracted serum samples (Michopoulos et al., 2011) or have validated oxytocin ELISAs for rodent species using raw plasma samples (Kramer et al., 2004). However, two papers to date have directly tested the benefits of extracting plasma samples prior to analysis, with both recommending the use of solid phase extraction with C18 Sep-Pak columns (Bachem, San Carlos, CA) as the best method to gain accurate detection levels for oxytocin in plasma (Szeto et al., 2011; Cool and DeBrosse, 2003). Szeto et al. (2011) report no correlation between oxytocin concentrations measured in raw plasma and those in extracted plasma, and call for further work to be carried out in order to develop reliable methods for oxytocin analysis. Additionally the large discrepancy between reported oxytocin concentrations detected in raw and extracted plasma (>100 pg/ml and <30 pg/ml, respectively, reviewed in Szeto et al., 2011) make comparisons across studies with different methodologies impossible and interpretation of which values most accurately reflect true oxytocin concentrations difficult.

This study investigates oxytocin plasma concentrations for the first time in two marine mammal species, the grey seal and harbour seal, and assesses how the measured concentration of oxytocin in plasma is affected by the type of anticoagulant used during collection, the handling process of the individual being sampled and subsequently whether the plasma is extracted prior to analysis. The relationship between raw and extracted oxytocin concentrations detected was also investigated in order to determine whether one can be used to accurately predict the other. This work was conducted during the development of the best protocol for detecting oxytocin accurately in phocid seals and was conducted as part of a larger study on phocid reproductive behaviour and physiology.

2. Methods

2.1. Ethics statement

This study used captive harbour and grey seals held under UK Home Office licence at the Sea Mammal Research Unit in St Andrews, Scotland. Capture and handling procedures were performed under Home Office project licence #60/4009 and conformed to the UK Animals (Scientific Procedures) Act, 1986. All research was approved ethically by the University of St Andrews Animal Welfare and Ethics Committee.

2.2. Study animals

Three seals provided blood samples for the comparison of vacutainer type and extraction protocol. Samples were collected over the period the seals were within the facility. The first was an adult male harbour seal of unknown age, brought into the facility on 10.02.11 from the Eden River, Scotland and released in November 2011. The other two were immature grey seals of known age, one male and one female. Both of these were born in November of the 2011 breeding season on the Isle of May, Scotland and brought

into captivity on 06.12.11 at approximately one month of age. The female was released in October 2012 and the male in December 2012. Additionally, a two-year old captive male harbour seal from the Moray Firth, Scotland kept from 01.10.10 until 29.10.11, was used to generate a dataset on the clearance rate of oxytocin in phocid seals using extracted and un-extracted samples.

2.3. Plasma sampling and analysis

Plasma samples were taken at approximately monthly intervals throughout the period of captivity ($n = 27$). A sample collected using heparin vacutainers was collected in all 27 sampling opportunities; however during two of the sampling opportunities it was not possible additionally to collect an EDTA sample, giving 25 matching EDTA plasma samples and a total number of 52 samples to compare to each other. To obtain a plasma sample, animals were captured and physically restrained, additionally animals over eight months of age were chemically immobilised using Zoletil® (Virbac). Samples were drawn from the extradural vein into either 10 ml lithium heparin or EDTA vacutainers without addition of aprotinin and stored on ice until they could be spun and frozen at −20 °C (CS Carter 2010 personal communication). While the ELISA protocol recommends freezing samples at −70 °C, this work was part of a larger project to determine the viability of samples obtained from a field site where only a −20 °C freezer was available. This study attempted to obtain all samples within five minutes of the initial disturbance to the animal as the reported short half life of oxytocin in plasma (Cool and DeBrosse, 2003) would suggest that slow sampling would not give a representative sample. The time taken to obtain a sample, and if chemical immobilisation was used, was recorded to account for these variables in the analysis. At each sampling event four 10 ml vacutainers of plasma (two of each type) were taken. Plasma was then analysed for oxytocin using an ELISA (Assay Designs Inc., Ann Arbor, MI, USA) with each sample undergoing solid-phase extraction using Sep-Pak C18 columns (Szeto et al., 2011; Cool and DeBrosse, 2003) prior to analysis following the manufacturer's instructions with the following modifications. First it was necessary to extract from 3 ml of plasma to allow use of the most sensitive part of the assay curve for determining concentrations of oxytocin. Second all samples had to be centrifuged after acidification for thirty minutes rather than fifteen; otherwise there was a risk that the plasma samples would block the Sep-Pak columns used to extract the peptide due to the high lipid and albumin concentrations present in phocid plasma (Hall, 1998). Extracts were then frozen until analysed, whereupon they were rehydrated according to the assay instructions and run on the ELISA plate. Both raw plasma and extracted plasma were analysed for all 52 samples collected during analysis, giving a total sample size of 104 samples for this study. The plate was read using a BioTek ELx800 reader and the standard curve and assay results for all plates were then fitted using the calibFit package (Haaland et al., 2011) in R version 2.9.2 (R Development Core Team, 2008). Recovery rates for the extraction and ELISA procedure were 107% ($n = 10$), intra-assay coefficient of variance for this assay was 4.6% and inter-assay coefficient of variance over the seven plates used in this study was 4%.

2.4. Clearance trial

For the duration of the 1 hour trial, the study animal was captured and chemically immobilised using Zoletil® (Virbac). A baseline sample was drawn from the extradural vein into 10 ml lithium heparin vacutainers, and then 3.96 μg oxytocin (0.022 ml of 0.18 mg/ml Oxytocin-S, Intervet UK Ltd. with 0.128 ml saline solution) was injected intravenously to create a hormone spike in the plasma. Serial samples were then taken every minute for the first five minutes of the trial, then every five minutes until 20 minutes post-injection, and finally every ten minutes until one hour post-injection. Samples were then stored, spun, frozen and analysed as described above, generating clearance curves for both extracted and raw versions of the same samples. The plasma clearance rate (CL) for both raw and extracted plasma samples from the same clearance trail were calculated using the following equation (Morin et al., 2008):

$$\mathrm{CL} = \frac{D}{\mathrm{AUC}}$$

where area under curve (AUC) is the area under the curve generated in the GAMs detailed below describing the relationship between plasma oxytocin concentrations and time post injection and D is the initial bolus of oxytocin injected in picograms (pg).

2.5. Statistical analysis

All analyses were performed using the statistical package R 2.15.0 (R Development Core Team, 2012). Generalised additive mixed models (GAM) (Wood, 2006a) were used to take into account variability in oxytocin generated by species, sex, the day of year the samples were taken, and the analysis protocol used (EDTA extracted, EDTA raw, heparin extracted and heparin raw). The models had a gamma error distribution with a log link and were fitted using the multiple generalised cross validation library mgcv (Wood, 2012). As any variation with 'day of year' (DOY) is unlikely to be linear, this was fitted as a smooth function (Wood, 2006b). Differences between species and sex in the seasonal pattern were examined by fitting additional smooth functions. The smoothing parameters were set by maximum likelihood to reduce the risk of overfitting associated with other methods (Wood, 2011). All other variables were fitted as fixed effects. Backwards stepwise elimination was carried out by using the Akaike information criterion (AIC) to identify the best model of the data and QQ and residual plots were examined to check the adequacy of the models. On this basis, all smooths were removed from the model, and DOY was removed entirely, simplifying the model into a generalised linear model (GLM) and leaving three fixed effects (species, sex and analysis protocol) which produced the best performing model.

Two separate GLMs were used to investigate any relationship between time taken to obtain a plasma sample, the use of chemical immobilisation or physical restraint and vacutainer type on the oxytocin concentrations detected in raw and extracted plasma. This could not be included in the first GLM as some values were missing from one individual's data, which would have reduced the number of samples included in the analysis above. The time in minutes it took to obtain a plasma sample from the initial disturbance to the individual, the type of vacutainer used for sample collection, what form of restraints (physical or chemical) were used for the procedure and an interaction term for sampling time/restraint type were fitted as fixed effects and a gamma error distribution with a log link was used. Backwards stepwise elimination was carried out by using the Akaike information criterion (AIC) to identify the best model of the data and QQ and residual plots were examined to check the adequacy of the models.

To investigate any correlations between oxytocin concentrations detected in extracted and raw plasma samples ($n = 52$), two GLM was used to model the relationship between the two measures taken with heparin ($n = 27$) and EDTA ($n = 25$) vacutainers separately and to predict extracted values of oxytocin from concentrations detected in raw samples. All GLM fits were fitted with gamma error distributions with log links and were checked with diagnostic plots and AIC scores to determine the best fits for the data.

Non-linear and GAM approaches were investigated to quantify the clearance curves generated from the IV oxytocin injection. The GAM approach was determined to be the most suitable for comparing the quality of the two clearance curves, and GAMs were made

Table 1
Fixed effect variables from the GLM of the effect of analysis protocol on detected oxytocin concentration, their estimates, standard errors and *p* values.

GLM variable	Estimate	Standard error	*p*-Value
Analysis protocol (heparin extracted)	0.03	0.087	0.71
Analysis protocol (heparin raw)	3.66	0.087	<0.001
Analysis protocol (EDTA raw)	4.21	0.088	<0.001
Sex	−0.18	0.072	0.012
Species	−0.53	0.077	<0.001

Table 2
Mean concentrations of oxytocin detected (pg/ml) in phocid plasma according to vacutainer type used to collect the sample and extraction protocol with standard errors.

	EDTA vacutainers	Heparin vacutainers
Extracted plasma	8.1 ± 0.6	8.3 ± 0.66
Raw plasma	543.2 ± 43.6	300.9 ± 19.6

for both extracted results and raw results. Both used one fixed effect (minutes post oxytocin injection) and as this variable was not linear it was fitted as a smooth function (Wood, 2006b) with smoothing parameters set by maximum likelihood (Wood, 2011).

3. Results

3.1. Causes of variability in oxytocin detection in seals

Of the variables tested, the GLM model showed that analysis protocol ($n = 104$ GLM, $F_{3,101} = 934.8$, $p < 0.001$) sex ($n = 104$ GLM, $F_{1,103} = 0.011$, $p = 0.01$) and species ($n = 104$ GLM, $F_{1,103} = 46.9$, $p < 0.001$) significantly affected the oxytocin detected in samples (Table 1). There was no significant difference between extracted samples taken with EDTA (mean = 8.1 ± 0.6 pg/ml) or heparin (mean = 8.3 ± 0.6 pg/ml) vacutainers ($p = 0.71$). However there were significant differences between all extracted and raw plasma protocols regardless of vacutainer type ($p < 0.001$) and between samples analysed raw taken using EDTA (mean = 543.2 ± 43.6 pg/ml) and heparin (mean = 300.9 ± 19.6 pg/ml) vacutainers ($p < 0.001$) (Table 2).

3.2. The effect of sampling time and restraint protocol on oxytocin detected

No relationship was found between oxytocin concentration detected in extracted plasma and the use of chemical or physical restraint in the capture process ($n = 36$ GLM, $F_{1,35} = 0.73$, $p = 0.27$), time taken to obtain a sample from first contact ($n = 36$ GLM, $F_{1,35} = 1.03$, $p = 0.14$), the interaction term for time to sample and type of restraint ($n = 36$ GLM, $F_{1,35} = 2.41$, $p = 0.13$) or vacutainer type ($n = 36$ GLM, $F_{1,35} = 0.61$, $p = 0.39$) (Table 3).

There were significant differences in detected oxytocin concentrations in raw plasma with the time taken to obtain a sample from first contact ($n = 36$ GLM, $F_{1,35} = 0.62$, $p = 0.007$), the interaction term for time to sample and type of restraint (physical or chemical) ($n = 36$ GLM, $F_{1,35} = 0.73$, $p = 0.01$) and vacutainer type ($n = 36$ GLM, $F_{1,35} = 28.8$, $p < 0.001$) (Table 4).

Table 3
Fixed effect variables from the GLM of the effect of time to sample, the type of restraint and vacutainer type on detected oxytocin concentrations in extracted plasma, their estimates, standard errors and *p* values.

GLM variable	Estimate	Standard error	*p*-Value
Time to obtain sample	0.28	0.18	0.14
Type of restraint	0.34	0.31	0.27
Vacutainer type	0.09	0.11	0.39
Interaction term between time to sample and type of restraint	−0.29	0.18	0.13

Table 4
Fixed effect variables from the GLM of the effect of time to sample, the type of restraint and vacutainer type on detected oxytocin concentrations in raw plasma, their estimates, standard errors and *p* values.

GLM variable	Estimate	Standard error	*p*-Value
Time to obtain sample	−0.53	0.18	0.007
Type of restraint (chemical)	−0.39	0.31	0.21
Vacutainer type (heparin)	−0.57	0.11	<0.001
Interaction term between time to sample and type of restraint	0.5	0.18	0.01

3.3. Correlations between oxytocin detected in raw and extracted plasma

No relationship was found between oxytocin concentrations detected in raw and extracted plasma samples taken with heparin ($n = 27$ GLM, $F_{1,26} = 1.24$, $p = 0.25$) or EDTA vacutainers ($n = 25$ GLM, $F_{1,24} = 2.47$, $p = 0.12$) (Fig. 1). Furthermore, when the best model was used to generate predicted oxytocin concentrations in extracted samples from a subset of raw values, the resulting dataset (mean = 2.1 ± 0.008 pg/ml) was significantly different from the true values (mean = 8.2 ± 0.4 pg/ml) when analysed using a Welch two sample *T* test ($t(15) = 8.2$, $p < 0.001$).

3.4. Clearance rate of oxytocin calculated using raw and extracted samples

Both GAM models showed that in raw and extracted plasma there was a significant decline in oxytocin over time towards the basal level ($n = 13$ in each GAM, for both $p < 0.001$). However the oxytocin values detected using raw plasma were much less successful at producing an accurate clearance curve compared to one generated with extracted plasma (percentage deviance explained: raw plasma: 30.7%, extracted plasma: 85.2%). In addition the confidence intervals were much wider around the fitted curve for the

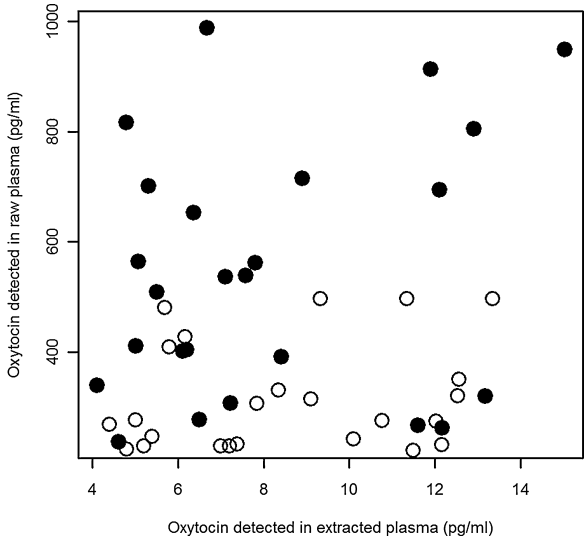

Fig. 1. Scatter plot showing the oxytocin concentrations detected in plasma samples that were analysed both raw and with extraction prior to assay. Samples collected in EDTA vacutainers are shown in black (●) and samples collected in heparin vacutainers are shown in white (○).

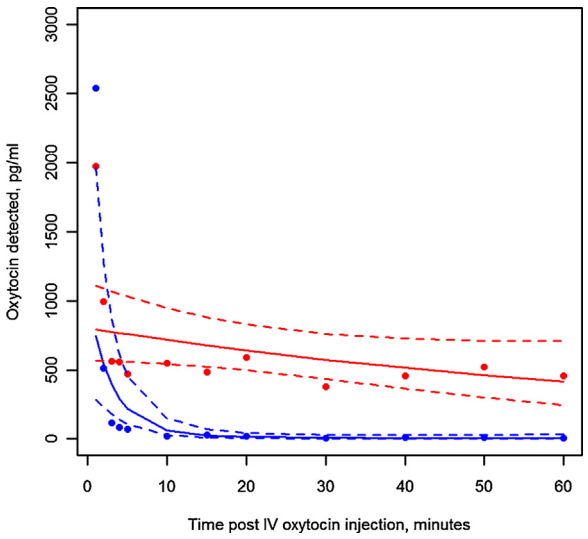

Fig. 2. Clearance curves for one individual given a single IV oxytocin dose, generated using both raw (red, the top solid line) and extracted (blue, the bottom solid line) plasma, with confidence intervals (dashed). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

raw results (Fig. 2). The plasma clearance rate calculated using the curve generated with extracted plasma was 0.035 L/min/kg, while the plasma clearance rate calculated using the curve generated with raw plasma was 0.004 L/min/kg.

4. Discussion

This study has determined successfully for the first time plasma oxytocin concentrations in two novel mammalian species, the grey seal and harbour seal. Furthermore, it has demonstrated clear and substantial differences in the oxytocin concentrations measured in phocid plasma samples depending on the protocol used to collect and analyse them. The largest differences were between raw and extracted plasma: raw samples produced oxytocin measures several hundreds times higher than extracted plasma. Importantly, no relationship was detected between the raw and extracted oxytocin concentrations. Such a relationship would be necessary for the prediction of one based on the other. Samples analysed raw were significantly influenced by the time it took to obtain a blood sample and by the type of restraints used, while extracted samples were not affected by these sources of variation. Finally samples taken with EDTA vacutainer tubes had significantly higher concentrations of oxytocin than heparin tubes when run raw on a plate, but when extracted there was no difference between the two. Therefore the design of a study protocol will have a major impact on the results generated, which must be taken into account when analysing and interpreting the data.

4.1. Raw versus extracted plasma

The results show clearly that the concentration of oxytocin detected in raw plasma samples is substantially higher than that in extracted samples (mean oxytocin extracted EDTA: 8.1 pg/ml, extracted heparin: 8.3 pg/ml, raw EDTA: 543.2 pg/ml, raw heparin: 300.9 pg/ml). These concentrations represent the first published data on plasma oxytocin concentrations found in a phocid seal, or any marine mammal species, and are comparable to both extracted and raw plasma concentrations reported in the literature for other mammalian species. Humans have been reported to have basal plasma oxytocin concentrations ranging between 0.1–23 pg/ml in extracted plasma and 99–405 pg/ml in raw plasma (reviewed in Szeto et al., 2011) and rats have mean oxytocin concentrations of 6.8 pg/ml in extracted plasma (Langraf, 1981) and concentrations ranging from 78.9–580 pg/ml in raw plasma (Carter et al., 2007; Martínez-Lorenzana et al., 2008). Szeto et al. (2011) detected the same contrast in concentrations measured using raw and extracted plasma while evaluating radioimmunoassay (RIA) and ELISA protocols for oxytocin analysis and used high performance liquid phase chromatography (HPLC) to investigate the components of raw plasma, enabling the identification of several oxytocin immunoreactive species in raw plasma. This accounts for the large difference between the concentrations of oxytocin measured in raw and extracted samples, and at least one of the additional immunoreactive species was identified as a degradation product of oxytocin. It is vital that any studies using raw plasma as the medium for analysis take this into account, as any results obtained will not be a reflection of oxytocin alone, but rather of an unknown number of other metabolic processes generating these reactive molecules in the blood.

The potential differences a study can incur in their results just by using raw or extracted plasma is well illustrated by the different plasma clearance rates generated from the clearance trial in this study. The plasma clearance rate for seals detected with extracted plasma (0.035 L/min/kg) is comparable to the existing values published for the clearance rate of oxytocin in other mammalian species detected with RIAs (0.027 L/kg/min in human men and 0.021 L/min/kg in human women (Leake et al., 1980), between 0.025 and 0.085 L/min/kg in rats depending on dose (Morin et al., 2008), and 0.016 L/min/kg in baboons (Kowalski et al., 1998)). However the plasma clearance rate generated by the raw plasma data (0.004 L/min/kg) is much slower than the rate from extracted samples and other published work due to the high AUC value generated from the raw plasma clearance curve (Fig. 2).

A difference between the two concentrations detected does not immediately rule out using information about one to infer the concentration of the other. If the other reactive species measured in the ELISA in raw samples are products of oxytocin metabolism then it could be theorised that raw levels would still give an indication of an individual's oxytocin activity in the blood. There have been studies that detected such a correlation, such as Michopoulos et al. (2011) and others that found no such link, such as Szeto et al. (2011). The current study was not able to find any correlation between the raw and extracted samples, and in addition found that regression models built on such data had poor predictive power for generating accurate extracted oxytocin levels from raw measurements. When analysing a set of serial samples taken during a clearance trial, the data generated from extracted samples produced a curve which accurately represented the decay rate. The data generated from raw samples, while still detecting an overall fall in oxytocin levels, could not have a clearance curve fitted to it that accounted for more than 30% of the variation in the data. Therefore our findings are in agreement with Szeto et al.'s work, that raw plasma cannot be used to accurately analyse oxytocin levels. However, Michopoulos et al. used serum rather than plasma for their analysis, which may have produced differences in the metabolites maintained or denatured during collection and storage. In addition the datasets used here and by Szeto et al. to generate the regressions and correlations are larger ($n = 52$ and $n = 39$, respectively) than the one used by Michopoulos et al. ($n = 11$) and therefore may have obtained a more representative dataset to work from. Michopoulos et al. also do not give details of the correlation model used to generate their results, preventing direct comparisons between the varying methods of statistical analysis used in the current study (GLM) and by Szeto et al. (Spearman rank correlation).

Therefore we caution that studies using raw plasma with the Assay Designs ELISA kit are unlikely to report oxytocin alone and additionally cannot accurately infer this data from their results.

4.2. Heparin versus EDTA vacutainers

The current study shows that if plasma is extracted prior to analysis, the particular anticoagulant in the vacutainer makes no difference to the concentration of oxytocin detected. This allows studies using extracted plasma to be comparable, regardless of vacutainer choice. However when analysing raw plasma, EDTA tubes contain almost double the concentration of oxytocin compared to heparin tubes collected samples (mean oxytocin EDTA: 543.2 pg/ml, heparin: 300.9 pg/ml). Other studies analysing different biological compounds in human samples have reported similar differences between the anticoagulants (Dong et al., 2010; Gonzalez-Covarrubias et al., 2013) while for some peptides there appears to be no difference (Varo et al., 2006). The protocol instructions for the Assay Designs ELISA do state that the chelators in EDTA might affect the activity of the conjugate used in the kit (Enzo Life Sciences Oxytocin ELISA kit Manual 2012). The conjugate is responsible for reacting with the *p*-nitrophenylphosphate substrate to generate a yellow colour, which indicates a low concentration of oxytocin as the intensity of the colour increases. If this function was impaired due to EDTA presence then no yellow colour would be generated and a false positive would occur. Heparin vacutainers work by potentiating the action of antithrombin (Chuang et al., 2001), which inactivates coagulation proteins rather than using chelators to bind ions, and this may account for the difference in results. This highlights the importance of fully investigating the consequences of any deviation from the kit manufacturer's instructions as the kit protocol recommends using EDTA tubes for collection with extracting prior to analysis. Therefore the discrepancy between the two tube types must be taken into account when designing a study, interpreting the results and comparing them across studies, and can be avoided completely if samples are extracted.

4.3. Influence of other variables

Several variables were analysed alongside protocol type to ensure all sources of variation in the dataset could be identified. The time to obtain a plasma sample and using physical or chemical restraints had no effect on the oxytocin detected in extracted samples, but significantly impacted on the concentrations detected in raw plasma. Oxytocin has been linked to restraint stress in rodents (Grippo et al., 2009) and is hypothesised to rise in response to high cortisol levels. However in previous studies only long term capture and restraint generated peaks in oxytocin (CS Carter, personal communication) and our results support this. As there were no significant changes in oxytocin concentration with time in extracted samples, it should be possible to obtain samples in future studies that allow detection of basal oxytocin concentrations from wild animals captured either manually or using a chemical immobiliser providing samples are extracted prior to analysis.

The individual's species and sex were both significant sources of variation in this study, while time of year was not. As this work includes few individuals, and no repeats of any species/sex combination were possible, stochastic differences between them are entirely plausible, and having access to a larger number of animals would have allowed us to examine this variation to determine its validity. Despite, there being significant variation in individual's oxytocin concentration, the differences generated by the four protocols are greater still and are present at the highest level of significance in the model.

Finally this study focused on the variation in oxytocin generated by extraction protocol and vacutainer choice, and did not specifically test for variation generated by differing storage temperatures or the use of aprotinin to stabilise the peptide. These two variables were kept consistent across the samples in this study, with all plasma stored at −20 °C with no addition of aprotinin. The coefficient of variance in this study remained consistently below 5% across two years of analysis, indicating that degradation of oxytocin in samples stored at −20 °C without the use of aprotinin was negligible. The influence of storage temperature and additional inhibitors on oxytocin concentration detected would be an area worthy of future study, as protocols in the literature vary on these points along with vacutainer type and use of extraction. The majority of studies published freeze at the recommended −70 °C with a minority using −20 °C or below (Scantamburlo et al., 2007; Yayou et al., 2010), yet this study and several others (Szeto et al., 2011; van der Post et al., 1997) indicate that oxytocin may be more stable in warmer storage temperatures than is thought currently. The use of aprotinin or any inhibitor post-sampling is extremely diverse in the literature, with as many studies using aprotinin (Feldman et al., 2010a,b; Skrundz et al., 2011; Weisman et al., 2013; Hoffman et al., 2012) as not (Scantamburlo et al., 2007; Yayou et al., 2010; Szeto et al., 2011; Deisenhammer et al., 2012; Hoge et al., 2012). The impact of these additional inconsistencies between studies in oxytocin analysis protocol would benefit from further investigation.

In conclusion, the protocol followed when collecting and analysing plasma for oxytocin does have a significant impact on the results generated and experimental design should be carefully considered when planning research in this area. The concentrations of basal plasma oxytocin in phocid seals are consistent with those detected in all mammal species studied to date in both extracted and raw plasma, and the clearance rate for this hormone in phocids is also comparable to that found in humans (Leake et al., 1980), rats (Morin et al., 2008) and primates (Kowalski et al., 1998). Therefore despite being novel species to this field, it appears that phocid seals represent a typical mammalian system in terms of plasma oxytocin. Therefore future work should use methods which generate reliable, repeatable measures of plasma oxytocin, as well as developing protocols for using other peripheral substrates such as saliva and urine. Due to their non-invasive nature, such mediums are becoming more popular but the methodologies to utilise them have their critics (Horvat-Gordon et al., 2005). Without a firm knowledge of what experimental variables researchers may inadvertently introduce into a study, it will be extremely difficult to decipher the role of this neuropeptide in human and animal social and reproductive systems.

Acknowledgements

We would like to thank Simon Moss, Ryan Milne, Alicia Widmer and everyone that assisted in caring for the seals during their time in captivity and in handling them during sampling events. We would also like to thank Ailsa Hall for her valuable advice on ELISA protocol and troubleshooting.

This work was conducted as part of a PhD, which was funded by the Natural Environment Research Council (NERC), UK and by SMRU Marine, St Andrews, UK. The funding sources had no role in the planning, execution, analysis or writing of this study.

References

Amico JA, Challinor SM, Cameron JL. Pattern of oxytocin concentrations in the plasma and cerebrospinal fluid of lactating rhesus monkeys (*Macaca mulatto*): evidence for functionally independent oxytocinergic pathways in primates. J Clin Endocrinol Metab 1990;71:1531–5.

Bales KL, van Westerhuyzen JA, Lewis-Reese AD, Grotte ND, Lanter JA, Carter CS. Oxytocin has dose dependant developmental effects on pair-bonding and alloparental care in female prairie voles. Horm Behav 2007;52:274–9.

Baskerville TA, Douglas AJ. Dopamine and oxytocin interaction underlying behaviours: potential contributions to behavioural disorders. CNS Neurosci Ther 2010;16:e92–123.

Carter CS, Pournajafi-Nazarloo H, Kramer KM, Ziegler TE, White-Traut R, Bello D, et al. Oxytocin: behavioural associations and potential as a salivary biomarker. Ann N Y Acad Sci 2007;1098::312–22.
Chuang YJ, Swanson R, Srikumar M, Raja M, Olson ST. Heparin enhances the specificity of antithrombin for thrombin and factor Xa independent of the reactive center loop sequence. J Biol Chem 2001;276:14961–71.
Crockford C, Wittig RM, Langergraber K, Ziegler TE, Zuberbühler K, Deschner T. Urinary oxytocin and social bonding in related and unrelated wild chimpanzees. Proc R Soc Lond B Biol Sci 2013;280:20122765.
Cool DR, DeBrosse D. Extraction of oxytocin and arginine–vasopressin from serum and plasma for radioimmunoassay and surface-enhanced laser desorption–ionization time-of-flight mass spectrometry. J Chromatogr B 2003;792:375–80.
Dale HH. On some physiological actions of ergot. J Physiol (Lond) 1906;34:163–206.
Deisenhammer EA, Hofer S, Scwitzer O, Defrancesco M, Lemmler G, Wildt L, et al. Oxytocin plasma levels in psychiatric patients with and without recent suicide attempt. Psychiatry Res 2012;200:59–62.
Devarajan K, Rusak B. Oxytocin levels in the plasma and cerebrospinal fluid of male rats: effects of circadian phase, light and stress. Neurosci Lett 2004;367:144–7.
Dong N, Dong J, Liu P, Xu L, Shi S, Wu Q. Effects of anticoagulants on human plasma soluble corin levels measured by ELISA. Clin Chim Acta 2010;14:1998–2003.
El-Masry N, Soliman A, Moety HA. Alterations of prolylendopeptidase, oxytocin and vasopressin activity in the plasma of autistic children. Curr Psychiatry 2010;17:31–7.
Feldman R, Gordon I, Schneiderman I, Weisman O, Zagoory-Sharon O. Natural variations in maternal and paternal care are associated with systematic changes in oxytocin following parent–infant contact. Psychoneuroendocrinology 2010a;35:1133–41.
Feldman R, Gordon I, Zagoory-Sharon O. The cross-generation transmission of oxytocin in humans. Horm Behav 2010b;58:669–76.
Gimpl G, Fahrenholz F. The oxytocin receptor system: structure, function and regulation. Physiol Rev 2001;81:629–83.
Gonzalez-Covarrubias V, Dane A, Hankemeier T, Vreeken RJ. The influence of citrate, EDTA and heparin anticoagulants to human plasma LC-MS lipidomic profiling. Metabolomics 2013;9:337–48.
Grippo AJ, Trhanas DM, Zimmerman RR, Porges SW, Carter CS. Oxytocin protects against negative behavioural and autonomic consequences of long term social isolation. Psychoneuroendocrinology 2009;34:1542–53.
Haaland P, Samarov D, McVey E. calibFit: statistical models and tools for assay calibration. R package version 2.1.0/r17; 2011 http://R-Forge.R-project.org/projects/calibfun/
Hall AJ. Blood chemistry and hematology of grey seal (*Halichoerus grypus*) pups from birth to postweaning. J Zoo Wildl Med 1998;29:401–7.
Heinrichs M, Baumgartner T, Kirschbaum C, Ehlert U. Social support and oxytocin interact to suppress cortisol and subjective responses to psychosocial stress. Soc Biol Psychiatry 2003;54:1389–98.
Hoffman ER, Brownley KA, Hamer RM, Bulik CM. Plasma, salivary and urinary oxytocin in anorexia nervosa: a pilot study. Eat Behav 2012;13:256–9.
Hoge EA, Lawson EA, Metcalf CA, Keshaviah A, Zak PJ, Pollack MH, et al. Plasma oxytocin immunoreactive products and response to trust in patients with social anxiety disorder. Depress Anxiety 2012;29:924–30.
Hollander E, Novotny S, Hanratty M, Yaffe R, DeCaria CM, Aronowitz BR, et al. Oxytocin infusion reduces repetitive behaviours in adults with autistic and Asperger's disorders. Neuropsychopharmacology 2003;28:193–8.
Holt-Lunstad J, Birmingham W, Light KC. The influence of depressive symptomatology and perceived stress on plasma and salivary oxytocin before, during and after a support enhancement intervention. Psychoneuroendocrinology 2011;36:1249–56.
Horvat-Gordon M, Granger DA, Schwartz EB, Nelson VJ, Kivlighan KT. Oxytocin is not a valid biomarker when measured in saliva by immunoassay. Physiol Behav 2005;84:445–8.
Kosfeld M, Heinrichs M, Zak PJ, Fischbacher U, Fehr E. Oxytocin increases trust in humans. Nature 2005;435:673–6.
Kowalski WB, Diveky L, Mehendale R, Parsons M, Wilson L Jr. Effect of pregnancy on the metabolic clearance rate and the volume of distribution of oxytocin in the baboon. Am J Physiol Endocrinol Metab 1998;274:791–5.
Kramer KM, Cushing BS, Carter CS, Wu J, Ottinger MA. Sex and species differences in plasma oxytocin using an enzyme immunoassay. Can J Zool 2004;82:1194–200.
Langraf R. Simultaneous measurement of arginine vasopressin and oxytocin in plasma and neurohypophyses by radioimmunoassay. Endokrinologie 1981;78:191–204.
Leake RD, Weitzman RE, Fisher DA. Pharmokinetics of oxytocin in the human subject. Obstet Gynecol 1980;56:701–4.
Leake RD, Weitzman RE, Fisher DA. Oxytocin concentrations during the neonatal period. Neonatology 1981;39:127–31.
Levine A, Zagoory-Sharon O, Feldman R, Weller A. Oxytocin during pregnancy and early postpartum: individual patterns and maternal–fetal attachment. Peptides 2007;28:1162–9.
Lévy F, Kendrick KM, Keverne EB, Piketty V, Poindron P. Intracerebral oxytocin is important for the onset of maternal behaviour in inexperienced ewes delivered under peridural anesthesia. Behav Neurosci 1995;106:427–32.
MacDonald K, MacDonald TM. The peptide that binds: a systemic review of oxytocin and its prosocial effects in humans. Harv Rev Psychiatry 2010;18:1–21.
Maestripieri D, Hoffman CL, Anderson GM, Carter CS, Higley JD. Mother–infant interactions in free-ranging rhesus macaques: relationships between physiological and behavioural variables. Physiol Behav 2009;96:613–9.
Martínez-Lorenzana G, Espinosa-López L, Carranza M, Aramburo C, Paz-Tres C, Rojas-Piloni G, et al. PVN electrical stimulation prolongs withdrawal latencies and releases oxytocin in cerebrospinal fluid, plasma and spinal cord tissue in intact and neuropathic rats. Pain 2008;140:265–73.
McCarthy MM. Oxytocin inhibits infanticide in female house mice (*Mus domesticus*). Horm Behav 1990;24:365–75.
McCullough ME, Churchland PS, Mendez AJ. Problems with measuring peripheral oxytocin: can the data on oxytocin and human behaviour be trusted? Neurosci Biobehav Rev 2013;37:1485–92.
Michopoulos V, Checchi M, Sharpe D, Wilson ME. Estradiol effects on behaviour and serum oxytocin are modified by social status and polymorphisms in the serotonin transporter gene in female rhesus monkeys. Horm Behav 2011;59:528–35.
Modahl C, Green L, Fein D, Morris M, Waterhouse L, Feinstein C, et al. Plasma oxytocin levels in autistic children. Soc Biol Psychiatry 1998;43:270–7.
Morin V, del Castillo JRE, Authier S, Ybarra N, Otis C, Gauvin D, et al. Evidence for nonlinear pharmacokinetics of oxytocin in anesthetized rat. J Pharm Pharmaceut Sci 2008;11:12–24.
Moscovice LR, Ziegler TE. Peripheral oxytocin in female baboons relates to estrous state and maintenance of sexual consortships. Horm Behav 2012;62:592–7.
Neumann ID, Landgraf R. Balance of brain oxytocin and vasopressin: implications for anxiety, depression, and social behaviours. Trends Neurosci 2012;35:649–59.
Nagasawa M, Kikusui T, Onaka T, Ohta M. Dog's gaze at its owner's urinary oxytocin during social interaction. Horm Behav 2009;56:434–41.
Neumann ID. Brain oxytocin: a key regulator of emotional and social behaviours in both females and males. J Neuroendocrinol 2008;20:858–65.
Odendaal JS, Meintjes RA. Neurophysiological correlates of affiliative behaviour between humans and dogs. Vet J 2003;165:296–301.
Popik P, Vetulani J, Van Ree JM. Low doses of oxytocin facilitate social recognition in rats. Psychopharmacology 1992;106:71–4.
Prakash BS, Vijay P, Kliem H, Kulozik U, Meyer HHD. Determination of oxytocin in milk of cows administered oxytocin. Anal Chim Acta 2009;636:111–5.
R Development Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistics Computing, Vienna, Austria. 2008 http://www.R-project.org
Ross HE, Young LJ. Oxytocin and the neural mechanisms regulating social cognition and affiliative behaviour. Front Neuroendocrinol 2009;30:534–47.
Scantamburlo G, Hansenne M, Fuchs S, Pitchot W, Marechal P, Pequeux C, et al. Plasma oxytocin levels and anxiety in patients with major depression. Psychoneuroendocrinology 2007;32:407–10.
Skrundz M, Bolten M, Nast I, Hellhammer DH, Meinlschmidt G. Plasma oxytocin concentration during pregnancy is associated with development of postpartum depression. Neuropsychopharmacology 2011;36:1886–93.
Szeto A, McCabe PM, Nation DA, Tabek BA, Rossetti MA, McCullough ME, et al. Evaluation of enzyme immunoassay and radioimmunoassay methods for the measurement of plasma oxytocin. Psychosom Med 2011;73:393–400.
Uvnäs-Moberg K. Oxytocin may mediate the benefits of positive social interaction and emotions. Psychoneuroendocrinology 1998;23:819–35.
van der Post JAM, van Buul BJA, Hart AAM, van Heerikhuize JJ, Pesman G, Legros JJ, et al. Vasopressin and oxytocin levels during normal pregnancy: effects of chronic dietary sodium restriction. J Endocrinol 1997;152:345–54.
Varo N, Nuzzo R, Natal C, Libby P, Schönbeck U. Influence of pre-analytical and analytical factors on soluble CD40L measurements. Clin Sci 2006;111:341–7.
Weisman O, Zagoory-Sharon O, Schneiderman I, Gordon I, Feldman R. Plasma oxytocin distributions in a large cohort of women and men and their gender-specific associations with anxiety. Psychoneuroendocrinology 2013;38:694–701.
Wood S. Generalized additive models: an introduction with R. Florida, USA: Chapman and Hall/CRC; 2006a.
Wood S. Low-rank scale-invariant tensor product smooths for generalized additive mixed models. Biometrics 2006b;62:1025–36.
Wood S. Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. J R Stat Soc B 2011;73:3–36.
Wood S. http://cran.r-project.org/web/packages/mgcv/index.html
Yayou K, Ito S, Yamamoto N, Kitagawa S, Okamura H. Relationship of stress responses with plasma oxytocin and prolactin in heifer calves. Physiol Behav 2010;99:362–9.